B cell modulation in atherosclerosis

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Bidirectional effects of IL-10^{+} regulatory B cells in *Ldlr*$^{-/-}$ mice

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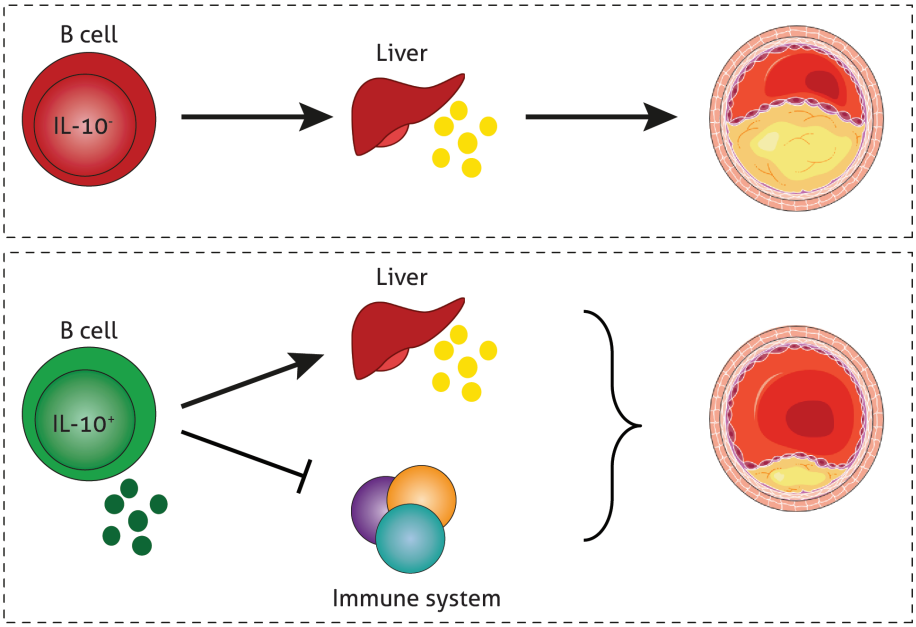
Abstract

Background and aims: Limiting the pro-inflammatory immune response is critical for the treatment of atherosclerosis. Regulatory B cells (Bregs) can modulate the immune response through interleukin-10 (IL-10). Current data regarding Bregs and atherosclerosis is scarce and conflicting.

Methods: In this study we investigated the frequency of IL-10$^+$ B cells during the development of atherosclerosis in low-density lipoprotein receptor-deficient (*Ldlr*$^{-/-}$) mice and studied the effect of adoptive transfer of IL-10$^+$ B cells on atherosclerosis.

Results: We found a very strong inverse correlation between atherosclerosis severity and the frequency of IL-10$^+$ B cells. This effect was cholesterol-independent and observed in spleen, draining lymph nodes and peritoneal cavity. To directly assess the effects of IL-10$^+$ B cells on atherosclerosis, we expanded IL-10$^+$ B cells *ex vivo* with anti-CD40 and selected pure and viable IL-10-secreting B cells and IL-10$^-$ B cells and adoptively transferred them to *Ldlr*$^{-/-}$ mice, respectively. Whereas IL-10$^-$ B cells were strongly atherogenic compared to control-treated mice, IL-10$^+$ B cells did not affect lesion size. Adoptive transfer of IL-10$^+$ B cells strongly reduced circulating leukocyte numbers and inflammatory monocytes. In addition, they decreased CD4$^+$ T cell activation and increased IL-10$^+$ CD4$^+$ T cell numbers. Interestingly, both IL-10$^+$ and IL-10$^-$ B cells exacerbated serum cholesterol levels and resulted in fatty livers, which potentially masked the beneficial effects of IL-10$^+$ B cells on atherosclerosis.

Conclusions: These findings underscore the strong immune-regulating function of IL-10$^+$ B cells and provide additional incentives to explore effective strategies that expand IL-10$^+$ B cells in atherosclerosis.



Graphical abstract.

Introduction

Cardiovascular disease remains a major global health problem and is mainly caused by atherosclerosis. Atherosclerosis is characterized by the build-up of lipids in the vascular wall which initiates a strong immune response, hence its classification as an autoimmune-like disorder[1]. At present, most treatment options are aimed at lipid lowering and do not address the ongoing immune response which perpetuates the disease. The recent success of the CANTOS trial strongly encourages research into novel therapeutic strategies that limit the immune reaction[2].

In the last decade, suppression of the immune reaction by regulatory B cells (Bregs) has received a lot of attention[3]. Bregs mediate the immune system primarily through interleukin-10 (IL-10) and it has been demonstrated that an imbalance in the number of Bregs strongly associates with multiple auto-immune disorders[4,5], including coronary artery disease[6]. In experimental models of auto-immune disorders, cellular therapy of Bregs have shown great potential, primarily via the induction of IL-10^{+} regulatory T cells[7–9]. Furthermore, it has been well-documented that IL-10 is protective in atherosclerosis[10]. However, there is a scarcity of research into Bregs and experimental atherosclerosis and the present data is conflicting[11–15]. While two groups report an atheroprotective effect of specific Breg subtypes[11,13], another study

using a mixed bone-marrow chimera model showed that B cell-derived IL-10 did not affect atherosclerosis[12]. These contradicting data clearly warrant more research into the contribution of Bregs to atherosclerosis.

A major hurdle in Breg research is the lack of a clearly defined immunophenotype. At present, there is a growing number of distinct Breg phenotypes defined by extracellular markers[7,16–21]. Most research up to date has focused on these Breg subset. However, IL-10$^+$ B cells are not limited to these subtypes and can be found within all B cell subsets, independent of their lineage or maturation and activation state[22]. Hence, IL-10 secretion is the primary mediator of immune suppression and remains the unique marker to classify Bregs. We thus aimed to determine in detail the role of IL-10$^+$ B cells in atherosclerosis by studying the frequency and status of IL-10$^+$ B cells during disease progression in low-density lipoprotein receptor-deficient mice (*Ldlr*$^{-/-}$) mice and by using an adoptive transfer model with viable IL-10-secreting B cells.

Material and methods

Animals

Male *Ldlr*$^{-/-}$ mice were bred in house and were kept under standard laboratory conditions. Mice were fed a normal chow diet or a Western-type diet (WTD) containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, Witham, Essex, UK). Diet and water were provided ad libitum. All injections were administered i.v. to the lateral tail vein in a total volume of 100 µl. During the experiments, mice were weighed, and blood samples were obtained by tail vein bleeding. At the end of experiments, mice were anaesthetized by a subcutaneous injection of a cocktail containing ketamine (40 mg/mL), atropine (50 µg/mL), and sedazine (6.25 mg/mL). Mice were exsanguinated by femoral artery transection followed by perfusion with PBS through the left cardiac ventricle. All animal work was approved by the Leiden University Animal Ethics Committee and the animal experiments were performed conform the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

Isolation of IL-10+ B cells

Viable IL-10-secreting B cells were isolated from *Ldlr*$^{-/-}$ donor mice using a regulatory B cell isolation kit according to protocol (Miltenyi Biotec). In short, B cells were isolated from the spleens of donor mice by negative selection. Secondly, these B cells were stimulated for 72h with 5 µg/ml of an agonistic CD40 antibody (Enzo

Life Science, clone FGK45). To stimulate active IL-10-secretion, B cells were further stimulated with LPS (50 µg/ml, *Escherichia coli* serotype O111:B4), PMA (50 ng/ml) and ionomycin (500 ng/ml) and labeled with an IL-10-catching antibody. Subsequently, the IL-10-secreting B cells were labelled with an IL-10 antibody conjugated to phycoerytrhin (PE) and finally isolated using magnetic PE-microbeads. The unlabeled cell fraction was also collected and determined as IL-10^{-} B cells. The isolated IL-10^{+} and IL-10^{-} B cells were consistently of a ≥90% purity.

Cell culture and cytokine determination

Isolated IL-10^{-} and IL-10^{+} B cells were cultured for 4 days with an agonistic CD40 antibody (5 µg/ml) after which IL-10 was measured in culture supernatants. For the T and B cell co-culture, naïve CD4^{+} T cells were isolated by magnetic selection according to the provided protocol (Miltenyi Biotec). 1x10^{5} CD4^{+} T cells were then plated in different ratios (1:2, 1:4, 1:8) with IL-10^{+} or IL-10^{-} B cells and stimulated with 5 µg/ml of plate-bound anti-CD3. After 72h of culture, cells were harvested and analyzed by flow cytometry. Concentrations of IL-10 in culture supernatants were measured using standard ELISA protocols (BD Bioscience).

Real-time quantitative PCR

RNA was extracted from isolated B cells by using Trizol reagent according to manufacturer's instructions (Invitrogen) after which cDNA was generated using RevertAid M-MuLV reverse transcriptase according to the instructions of the manufacturer (Thermo Scientific). Quantitative gene expression analysis was performed using Power SYBR Green Master Mix on a 7500 Fast Real-Time PCR system (Applied Biosystems). Gene expression of LIGHT (Forward: ATCTCACCAGGCCAACCCAGCA, Reverse: CCAGGCCAAGTCGTGTCTCCCATA) and lymphotoxin-alpha (Forward: CTGAAACCTGCTGCTCACCT, Reverse: ATGTCGGAGAAAGGCACGAT) was normalized to housekeeping genes (hydroxymethylbilane synthase, ribosomal protein 37 and glyceraldehyde 3-phosphate dehydrogenase).

Diet induced atherosclerosis

For the time-course experiment, age-matched (10-12 weeks old) *Ldlr*$^{-/-}$ mice were fed a WTD for 0, 3, 6 or 9 weeks (n=6/group). Mice were sacrificed at the same time-point after which the spleens were analyzed for the presence of IL-10^{+} B cells by flow cytometry. In a separate experiment, age-matched *Ldlr*$^{-/-}$ mice received a normal chow diet or WTD for 9 weeks (n=7/group). Multiple organs were harvested and processed for flow cytometry analysis. For the diet-switch experiment, *Ldlr*$^{-/-}$ mice were given either a WTD for 19 weeks, normal chow diet for 19 weeks or a WTD for 16 weeks and subsequently 3 weeks of normal chow diet (n=6/group).

These three weeks were determined by measuring the cholesterol levels during the switch to chow diet and the experiment was terminated when the cholesterol levels were normalized (data not shown). For the adoptive transfer experiment, at the start of WTD, 10-12 weeks old *Ldlr*$^{-/-}$ acceptor mice received 100 µl PBS or a cell suspension containing either 1x10^{6} IL-10^{-} or IL-10^{+} B cells (n=15/group). These injections were repeated at week 3 and 6 and mice were sacrificed after 9 weeks for analysis.

Flow cytometry

For the analysis of IL-10^{+} B cells, single cell suspensions were stimulated for 5 hours with LPS (50 µg/ml), PMA (50 ng/ml), ionomycin (500 ng/ml) and monensin (2 µM). For B10pro cells, B cells were first isolated with CD19-microbeads (Miltenyi Biotec) and subsequently stimulated for 48h with LPS (10 µg/ml) and/or an agonistic CD40 antibody (5 µg/ml). Secretion of cytokines by T cells was induced with stimulation of PMA, ionomycin and brefeldin A (5 µg/ml). For flow cytometry analysis, Fc receptors were blocked (2.4G2) and samples were stained with a dead/live marker (Fixable Viablity Dye) and anti-mouse fluorochrome-conjugated antibodies against B220 (RA3-6B2), CD19 (1D3), IL-10 (JES-16E3), CD4 (RM4-5), CD86 (GL1), CD69 (H1.2F3), CD25 (PC61.5), NK1.1 (PK136), CD11b (M1/70), Ly6-G (1A8), Ly-6C (HK1.4), CD62l (MEL-14), CD44 (IM7), IgM (II/41), IgD (11-26c), CD21 (7E9), CD23 (B3B4), CD24 (M1/69), TIM-1 (RMT1-4), CD1d (1B1), CD5 (53-7.3) or TNFα (MP6-XT22). All antibodies were purchased from Thermo Fisher Scientific or Becton Dickinson. FACS analysis was performed on a FACSCanto II (Becton Dickinson), and the acquired data were analyzed using FlowJo software. Gates were set according to unstimulated controls (only treated with monensin or brefeldin A) or to isotype and fluorescence minus one controls.

Serum measurements

During the experiment, blood was collected from the tail vein. After euthanasia, orbital blood was collected in EDTA-coated tubes. Whole blood cell counts were analyzed using the XT-2000iV hematology analyzer (Sysmex Europe GMBH, Norderstedt, Germany). Serum was acquired by centrifugation and stored at −20°C until further use. The total cholesterol levels in serum were determined using enzymatic colorimetric procedures (Roche/Hitachi, Mannheim, Germany). Precipath (Roche/Hitachi) was used as an internal standard. Lipoprotein profiles were determined by fractionation of 50 µL samples of serum using a Superose 6 column (3.2×300 mm, Smart-System; Pharmacia, Uppsala, Sweden) after which total cholesterol levels were determined in each fraction.

Liver lipid extraction and quantification

Triglycerides were extracted from liver tissue using Nonidet™ P 40 Substitute (Sigma-Aldrich Corp.). Liver tissue was homogenized with Nonidet™ P 40 Substitute, heated until 90°C and chilled on ice. This procedure was repeated once and subsequently insoluble material was removed by centrifugation. Cholesterol was extracted from liver tissue by the Folch extraction method[23]. Triglycerides and cholesterol concentration was determined by an enzymatic colorimetric assays.

Histology

To determine lesion size, cryosections (10 µm) of the aortic root were stained with Oil-Red-O and hematoxylin (Sigma-Aldrich). Sections with the largest lesion plus four flanking sections were analyzed for lesion size. Lipid content in liver sections was evaluated using the same Oil-Red-O staining. Collagen content in the lesion was assessed with a Masson's trichrome staining according to the manufacturers protocol (Sigma-Aldrich). Corresponding sections on separate slides were also stained for monocyte/macrophage content using a monoclonal rat IgG2b antibody (MOMA-2, 1:1000, AbD Serotec) followed by a goat anti-rat IgG-horseradish peroxidase antibody (1:100, Sigma-Aldrich) and color development.

Statistics

All data are expressed as mean ± SEM. Data were tested for significance using a Student's t-test for two normally distributed groups. Data from three groups or more were analyzed by an ordinary one-way ANOVA test followed by Tukey's post hoc test. When data from multiple time-points were tested, a repeated measures ANOVA was performed instead. Probability values of $p < 0.05$ were considered significant. All statistical analysis was performed using GraphPad Prism 7.0.

Results

IL-10^{+} B cells are significantly reduced during atherosclerosis development in *Ldlr*$^{-/-}$ mice

To examine the frequency of IL-10^{+} B cells during the development of atherosclerosis, we fed age-matched *Ldlr*$^{-/-}$ mice a Western type diet (WTD) for different time points (0, 3, 6 or 9 weeks) to induce various degrees of atherosclerosis. In general, two IL-10^{+} B cell types can be identified using flow cytometry; B10 and B10pro cells[24,25]. B10 cells readily secrete IL-10 after a short-term (5h) stimulation protocol (Fig. S1), while B10pro cells develop into IL-10-producing B cells after a long-term (48h) stimulation protocol with anti-CD40 and LPS. We found a gradual but sig-

nificant decrease of B10 cells in the spleen when mice were fed a WTD diet for longer periods (Fig. 1A). To assess whether the loss of IL-10$^+$ B cells correlated with disease severity, we measured the lesion size in aortic root sections and found a very significant inverse correlation between Bregs and atherosclerosis severity (Fig. 1B). Similarly, we observed an even stronger decrease in B10pro cells with increasing weeks of feeding the mice a WTD (Fig. 1C), which again highly correlated with increased atherosclerosis (Fig. 1D). In a separate experiment, we found that this effect was not spleen-specific but the loss of IL-10$^+$ B cells was also observed in the mediastinal lymph nodes draining from the aortic root and in the peritoneal cavity (Fig. 1E). In contrast, B cells in non-draining lymph nodes and in the circulation were

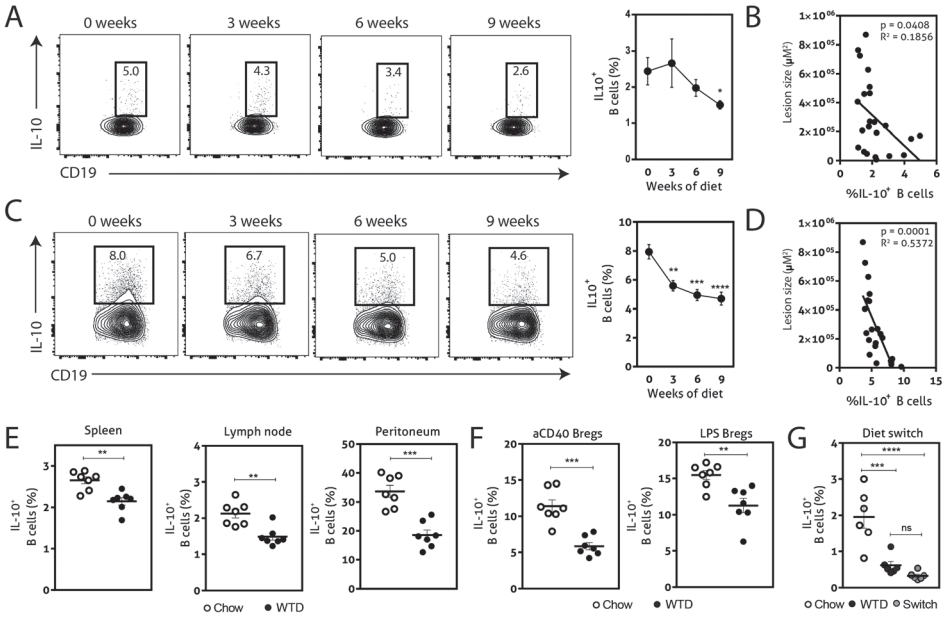


Figure 1. IL-10$^+$ B cells are significantly reduced during atherosclerosis development in *Ldlr*$^{-/-}$ mice. Age-matched *Ldlr*$^{-/-}$ mice were fed a Western type diet (WTD) or normal chow diet for varying periods and sacrificed at the same day. **(A)** IL-10$^+$ B cells in splenocytes were determined with flow cytometry after a short-term (5h) stimulation with LPS (50 µg/ml), PMA (50 ng/ml), ionomycin (500 ng/ml) and monensin (2 µM) (LPIM) and **(B)** correlated with lesion size in aortic root sections. **(C)** IL-10$^+$ B cells were determined after a long-term (48h) stimulation of isolated splenic B cells with LPS (50 µg/ml) and anti-CD40 (5 µg/ml) followed by LPIM stimulation and **(D)** correlated with lesion size in aortic root sections. **(E)** *Ldlr*$^{-/-}$ mice were fed a WTD or normal chow diet for 9 weeks after which IL-10$^+$ B cells were determined using LPIM stimulation in the spleen, lymph node and peritoneal cavity. **(F)** Identification of IL-10$^+$ B cells in mice fed a normal chow diet or a WTD for 9 weeks after stimulation of isolated splenic B cells with either LPS (50 µg/ml) or anti-CD40 (5 µg/ml) followed by LPIM stimulation. **(G)** *Ldlr*$^{-/-}$ mice were fed a normal chow diet for 19 weeks, a WTD for 19 weeks or a WTD for 16 weeks followed by a normal chow diet for 3 weeks (switch) to normalize cholesterol levels after which IL-10$^+$ B cells were determined in the spleen with LPIM stimulation. Data are shown as mean ± SEM (*p<0.05, ** p<0.01, ***p<0.001, ****p<.00001).

not affected by WTD-feeding (Fig. S2). We subsequently tested if a particular subset of regulatory B cells was affected during atherosclerosis development. This did not reveal a consistent reduction in specific subsets when we analyzed B cells in the spleen and lymph node (Fig. S3A-C). Along this line, we did not identify any differences in peritoneal subsets that pinpointed a particular B cell population that lost its IL-10 production after WTD diet feeding (Fig. S3D) We next wondered if the drastic reduction in B10pros was specifically a consequence of unresponsiveness to either LPS or anti-CD40. However, irrespective of the stimulus, flow cytometry revealed a highly significant decrease in the percentage of IL-10$^+$ B cells in the spleen of WTD-fed mice compared to chow mice (Fig. 1F). Since *Ldlr*$^{-/-}$ mice fed a WTD have increased levels of serum cholesterol, we investigated whether this was directly affecting the number of IL-10$^+$ B cells. Hence, we used a diet switch experiment by feeding *Ldlr*$^{-/-}$ mice either a normal chow diet, a WTD or a WTD followed by 3 weeks of a normal chow diet to normalize the cholesterol levels. We found that while the cholesterol levels in the diet-switched mice were fully normalized from 991 mg/dl to 240 mg/dl (data no shown), the loss of IL-10$^+$ B cells was not restored and remained significantly decreased compared to mice on a normal chow diet (Fig. 1G). Thus, IL-10$^+$ B cells showed a strong inverse correlation with atherosclerosis severity, but this was not directly correlated to cholesterol serum levels.

Adoptive transfer of IL-10$^+$ B cells leads to strong immune regulation

Given the strong decrease of IL-10$^+$ B cells during atherosclerosis progression, we wanted to explore strategies to restore the immune-regulating function of B cells. However, the low frequency and availability of IL-10$^+$ B cells severely limits the options of using them for cellular therapy. The practical difficulties can be overcome by either FACS-sorting Breg subpopulations based on extracellular markers[11,17] or to expand Bregs *ex vivo*, for example using anti-CD40 stimulation[8,26]. The drawback is that both of these options at best result in a B cell population that is an enriched mixture of IL-10$^+$ and IL-10$^-$ B cells. Since we found the greatest reduction in CD40-generated IL-10$^+$ B cells during atherosclerosis progression (Fig. 1F), we hypothesized that adoptive transfer of these cells would be most beneficial in the context of atherosclerosis. To directly assess the function of IL-10$^+$ B cells, we used a specialized kit to isolate viable IL-10-secreting B cells from anti-CD40 stimulated B cells that resulted in a 94.4% purity on average (Fig. S4). These IL-10$^+$ B cells displayed an IgM^{hi}IgD^{lo}CD21^{lo}CD24^{hi}CD1d^{hi}CD5$^+$ phenotype compared to IL-10$^-$ B cells (Fig. 2A), which highly corresponds with previously described IL-10$^+$ B cells from apolipoprotein E-deficient mice[24]. Furthermore, cytokine analysis revealed that while IL-10$^+$ B cells readily secrete IL-10 after several days of culture, we failed to detect IL-10 in supernatants of IL-10$^-$ B cells (Fig. 2B). Another major determinant of

regulatory B cells is their effect on CD4$^+$ T cell responses. In a co-culture experiment with CD4$^+$ T cells, our isolated IL-10$^+$ B cells significantly inhibited the development of TNFα$^+$ CD4 T cells. This inhibition was dependent on the B to T cell ratio and correlated with IL-10 levels in culture supernatants (Fig. 2C). Taken together, these data indicate that our culture protocol and isolation resulted in a highly pure population of IL-10$^+$ B cells with a clear regulatory phenotype and function. We adoptively transferred these IL-10$^+$ B cells to *Ldlr*$^{-/-}$ mice, which were subsequently fed a WTD for 9 weeks while PBS treatment and the negative fraction of the isolation (IL-10$^-$ B cells) were used as controls (Fig. S5).

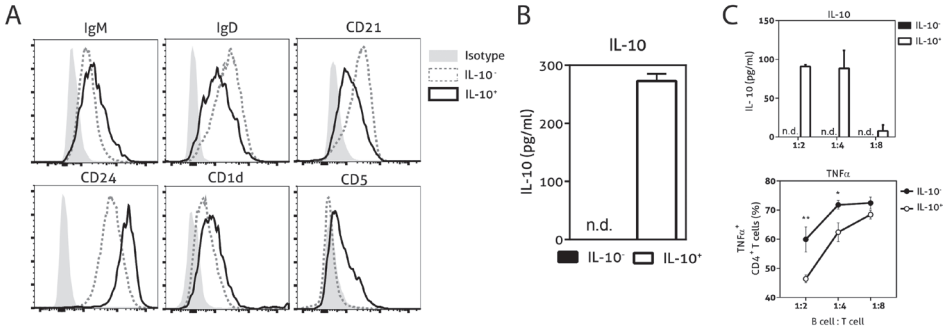


Figure 2. Characterization of IL-10$^+$ B cells. B cells were cultured with an agonistic CD40 antibody (5 μg/ml) for 72 hours after which they were stimulated with LPS (50 μg/ml), PMA (50 ng/ml) and ionomycin (500 ng/ml) to secrete IL-10. Subsequently IL-10$^-$ and IL-10$^+$ B cells were isolated. **(A)** Characterization of extracellular markers of IL-10$^-$ and IL-10$^+$ B cells with flow cytometry). **(B)** IL-10$^-$ and IL-10$^+$ B cells were cultured for 4 days with an agonistic CD40 antibody (5 μg/ml) after which IL-10 was measured in culture supernatants **(C)** Different ratios of IL-10$^-$ and IL-10$^+$ B cells were co-cultured with naïve CD4$^+$ T cells and stimulated with anti-CD3 (5 μg/ml). After 72 hours, IL-10 was measured in cultured supernatants with ELISA and TNFα$^+$ CD4 T cells were determined with flow cytometry.

Adoptive transfer of IL-10$^+$ B cells led to a very marked reduction in all circulating leukocytes compared to mice receiving PBS or IL-10$^-$ B cells (Fig. 3A). Monocytes contribute significantly to lesion formation and IL-10 is known to inhibit monocyte adhesion and affect the monocyte polarization[27,28]. Hence, we used flow cytometry to measure the number of inflammatory monocytes and found that mice treated with IL-10$^+$ B cells had a strong reduction in circulating inflammatory monocytes (Fig. 3B), indicating that adoptive transfer of IL-10$^+$ B cells led to a less atherosclerosis-prone phenotype of monocytes. Moreover, it is well-known that Bregs have strong effects on CD4$^+$ T cells[22]. Mice treated with IL-10$^+$ B cells showed a very strong increase in circulating IL-10$^+$ CD4$^+$ T cells compared to mice receiving PBS or IL-10$^-$ B cells (Fig. 3C). Similarly, we found that these circulating CD4$^+$ T cells showed a more naïve phenotype in mice treated with IL-10$^+$ B cells, while mice receiving IL-10$^-$ B cells demonstrated a

strong increase in effector memory CD4$^+$ T cells (Fig 3D). We also measured CD4$^+$ T cell activation in the lymph node draining from the aortic root and found a significant reduction in expression of CD69, CD25 and CD86 on CD4$^+$ T cells in mice treated with IL-10$^+$ B cells compared to mice receiving PBS or IL-10$^-$ B cells (Fig. 3E). This illustrated that the CD4$^+$ T cells are less activated in mice receiving IL-10$^+$ B cells compared to mice treated with PBS or IL-10$^-$ B cells. Taken together, these data clearly indicate that adoptive transfer of IL-10$^+$ B cell resulted in significant immune regulation.

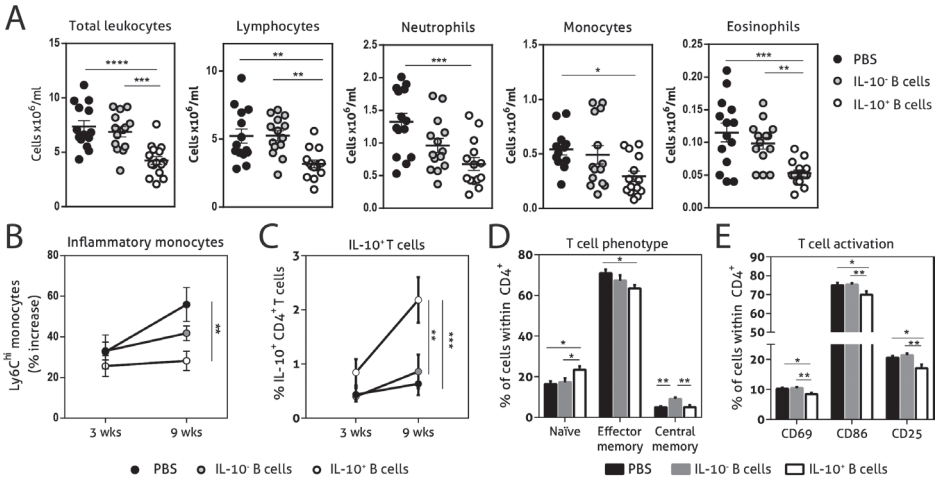


Figure 3. Adoptive transfer of IL-10$^+$ B cells leads to strong immune regulation. *Ldlr*$^{-/-}$ mice were put on a Western type diet for 9 weeks and received either three i.v. injections of PBS, IL10$^+$ or IL10$^-$ B cells (1x10^6 cells/injection). **(A)** Hematologic analysis of circulating white blood cells. Blood samples were analyzed with flow cytometry for (**B)** inflammatory monocytes (B220$^-$NK1.1$^-$ CD11b$^+$SSC^{lo}Ly-6G^{int}Ly-6C^{hi}), **(C)** IL-10$^+$CD4$^+$ T cells and **(D)** naïve (CD62l$^+$CD44$^-$), effector memory (CD62l$^-$CD44$^+$) or central memory (CD62l$^+$CD44$^+$) CD4$^+$ T cells. **(E)** Single cell suspensions of isolated lymph nodes were analyzed for activation markers on CD4$^+$ T cells with flow cytometry. Data are shown as mean ± SEM (*p<0.05, ** p<0.01, ***p<0.001, ****p<.00001).

IL-10$^-$ B cells are strongly atherogenic while IL-10$^+$ B cells do not affect lesion size

Considering the strong immune regulation after treatment with IL-10$^+$ B cells, we wondered what their effect on atherosclerosis development was. Hence, we measured lesion size and composition in the aortic root. We found that while treatment of mice with IL-10$^-$ B cells led to a drastic increase in atherosclerosis, treatment of mice with IL-10$^+$ B cells did not affect lesion size compared to PBS-treated mice (Fig. 4A). Similarly, lesion composition in terms of collagen and macrophage content did not differ between mice treated with PBS or IL-10$^+$ B cells. In contrast, lesions of mice that received IL-10$^-$ B cells showed a more advanced lesion phenotype with increased collagen and total macrophage area (Fig. 4B and 4C).

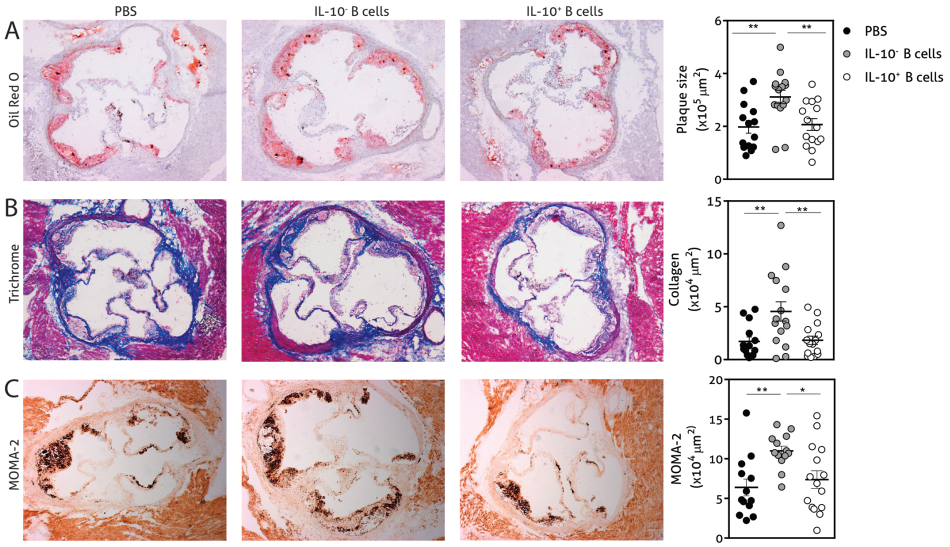


Figure 4. IL-10⁻ B cells are strongly atherogenic while IL-10⁺ B cells do not affect lesion size. *Ldlr*$^{-/-}$ mice were put on a Western type diet for 9 weeks and received either three i.v. injections of PBS, IL10^{+} or IL10^{-} B cells (1x10^{6} cells/injection). Representative pictures and quantification of aortic-root sections stained with **(A)** Oil Red O **(B)** Masson's trichrome staining and **(C)** MOMA-2. Data are shown as mean ± SEM (*p<0.05, ** p<0.01).

Adoptive transfer of both IL-10⁻ and IL-10⁺ B cells leads to exacerbated serum cholesterol levels

Given the strong evidence for an anti-atherogenic immune regulatory effect of IL-10^{+} B cells, we were surprised to find that adoptive transfer of IL-10^{+} B cells did not alter lesion size or composition. To explain this discrepancy, we explored other parameters, which could have masked the anti-atherogenic effects of IL-10^{+} B cells on the immune system. Considering the strong correlation of serum cholesterol levels with lesion size, we determined whether the cellular therapy had resulted in altered lipid levels. Indeed, we found differences in body weight (Fig. 5A) and an unexpected increase in total serum cholesterol levels in mice receiving IL-10^{+} as well as IL-10^{-} B cells (Fig. 5B), which was primarily caused by an increase in the levels of very-low density lipoprotein (Fig. 5C). We determined significant correlations of serum cholesterol levels and lesion size for mice treated with PBS or IL-10^{-} B cells, but this correlation was abolished in mice that received IL-10^{+} B cells (Fig. 5D). Since cholesterol is a key determinant for lesion development, this suggests that lipid-independent mechanisms interfered during the development of atherosclerosis. We further investigated the livers of mice treated with IL-10^{+} or IL-10^{-} B cells and found that these displayed increased Oil Red O staining compared to the PBS-treated mice (Fig. 5E). Moreover, we analyzed the lipid content of the livers and found a remark-

able increase in both cholesterol (Fig. 5F) and triglycerides (Fig. 5G) in mice that received B cell adoptive transfers compared to PBS-treated mice. Given the strong immune regulation in mice receiving IL-10^{+} B cells we believe that these effects have negated the atherogenic effects of increased cholesterol levels. In an effort to shed some light on the increased cholesterol levels, we measured the expression of lymphotoxin-alpha (LTα) and LIGHT on B cells after CD40-stimulation, since these molecules have previously been shown to regulate cholesterol levels in *Ldlr*$^{-/-}$ mice[29]. We found that CD40-stimulation of B cells significantly enhanced the expression of both LIGHT (Fig. S6A) and LTα (Fig. S6B). Our isolation protocol further required B cells to be stimulated with LPS, PMA and ionomycin and after this additional stimulus, expression of LIGHT and LTα remained high on B cells (Fig. S6A-B). Subsequently, we separated IL-10^{-} and IL-10^{+} B cells and measured the gene expression of LIGHT and LTα by qPCR and did not find any significant differences between IL-10^{-} and IL-10^{+} B cells (Fig. S6C). These findings might explain the increased cholesterol levels found in mice that received an adoptive transfer with IL-10^{-} or IL-10^{+} B cells.

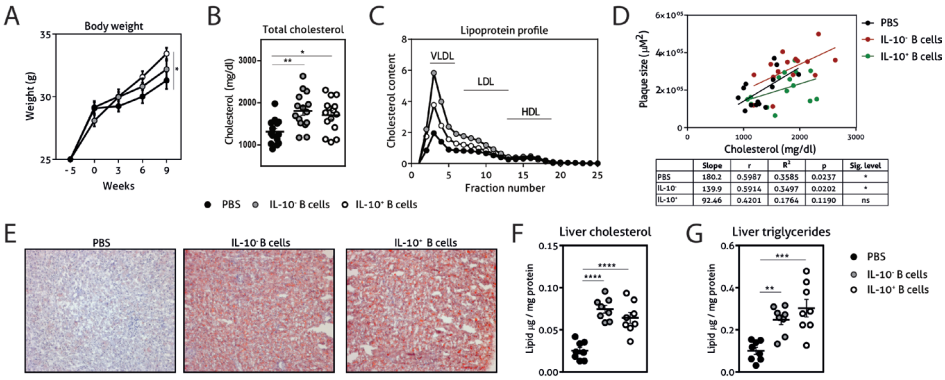


Figure 5. Adoptive transfer of both IL-10^{-} and IL-10^{+} B cells leads to exacerbated serum cholesterol levels. *Ldlr*$^{-/-}$ mice were put on a Western type diet for 9 weeks and received either three i.v. injections of PBS, IL10^{+} or IL10^{-} B cells (1x10^{6} cells/injection). (A) Body weight was assessed during the experiment. (B) Quantification of total serum cholesterol levels. (C) Characterization of the serum lipoprotein profile. (D) Correlations between serum cholesterol levels and plaque size in the aortic root. (E) Sections of liver tissue were stained with Oil-Red-O. (F) Quantification of extracted cholesterol content in the liver. (G) Quantification of extracted cholesterol content in the liver. Data are shown as mean ± SEM (*p<0.05, ** p<0.01, ***p<0.001, ****p<.00001).

Discussion

Since the role of Bregs in cardiovascular disease is currently under debate[11–13], we aimed to investigate the contribution of IL-10^{+} B cells to diet-induced atherosclerosis. IL-10^{+} B cells were drastically reduced during atherosclerosis development and

adoptive transfer of pure IL-10^{+} B cells limited the immune reaction. However, this was also associated with increased serum cholesterol levels and a fatty liver and this bidirectional effect prevented amelioration of atherosclerosis.

The increase in serum cholesterol levels after adoptive B cell transfer was a major unexpected finding of this study. There is a paucity of data examining the relation between immune cells and plasma cholesterol levels. While effects of dendritic cells[30] and T cells[29] have been described previously, a direct contribution of B cells to cholesterol homeostasis has not been recognized before. However, it has previously been shown that expression of LTα and LIGHT on CD4^{+} T cells is able to increase serum cholesterol levels[29]. Since it has also been shown that CD40-activation of B cells increases the expression of lymphotoxin on B cells[31,32], we measured the expression of LTα and LIGHT on B cells in our experiments. We found that our *ex vivo* stimulation protocol led to a considerable increase in extracellular expression of both proteins, which could potentially explain the exacerbated lipid levels found in this study. Despite the lack of a defined causal mechanism, these findings underscore that the immune response and plasma lipid levels are more interrelated in atherosclerosis than frequently thought. Additionally, the increased plasma lipids have undoubtedly influenced the atherosclerosis development in our study and could have masked the beneficial effects of IL-10^{+} B cells on atherosclerosis.

We did however find a strong atherogenic effect of IL-10^{-} B cells, which was not found using IL-10^{+} B cells. It is well-known that splenic B2 cells increase lesion size[33,34], in line with our findings using IL-10^{-} B cells, however we show here that pure splenic IL-10 producing B cells do not contain these atherogenic properties. Moreover, they limited the immune response and increased numbers of IL-10^{+} T cells. These findings corroborate with earlier data of an adoptive transfer study using CD21^{hi}CD23^{hi}CD24^{hi} B cells that both induced IL-10^{+} T cells and exerted vascular protection[11]. In contrast, another study investigating the role of B cell-derived IL-10 did not find any effect on atherosclerosis[12]. The authors used a mixed bone-marrow chimera model to establish a B cell-specific loss of IL-10, which did not influence lesion size. However, they also showed that IL-10 expression in the aorta was actually increased, possibly suggesting that IL-10 from other sources than B cells might compensate for the loss of B cell-derived IL-10[12]. Additional evidence for an atheroprotective role of Bregs was found by the observation that L-selectin deficiency decreased the aortic Breg cells and promoted atherosclerosis[13]. More recently, it was shown that induction of IL-10 producing B cells by a synergistic effect of BAFF and angiotensin II was associated with atheroprotection[33]. It has

also been shown that in human coronary artery disease Bregs have an impaired regulatory function[6].

Given the substantial reduction of IL-10^{+} B cells in atherosclerosis and the immune-regulating effects found in this study, we believe there is still ample evidence that IL-10^{+} B cells could protect against atherosclerosis. However, the lack of means to expand the number of Bregs *in vivo* still hampers this research. In this study we investigated a pure population of IL-10-secreting B cells, however the bidirectional effects induced by our culture protocol prevented an atheroprotective effect. In this respect, future research focusing on other strategies to induce Bregs in atherosclerosis remains of great interest.

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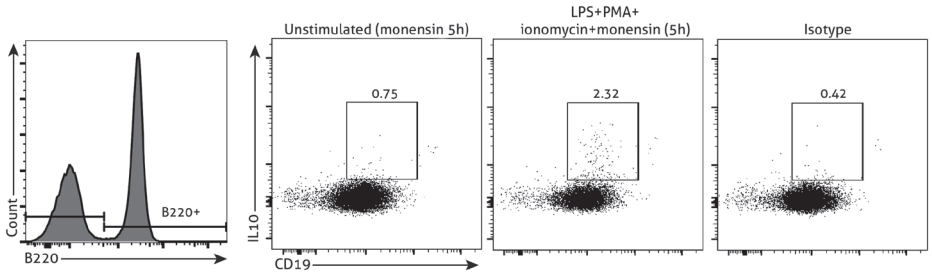
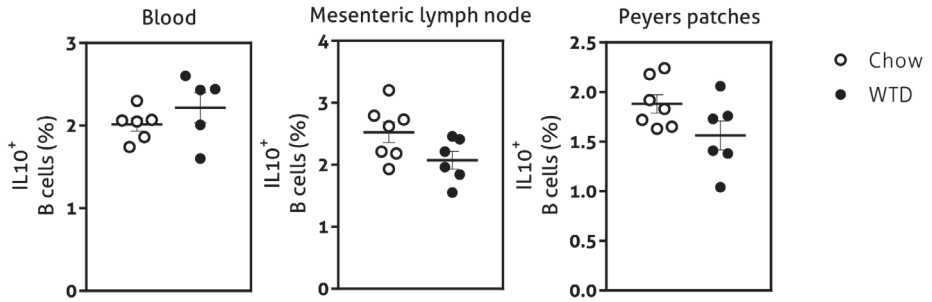
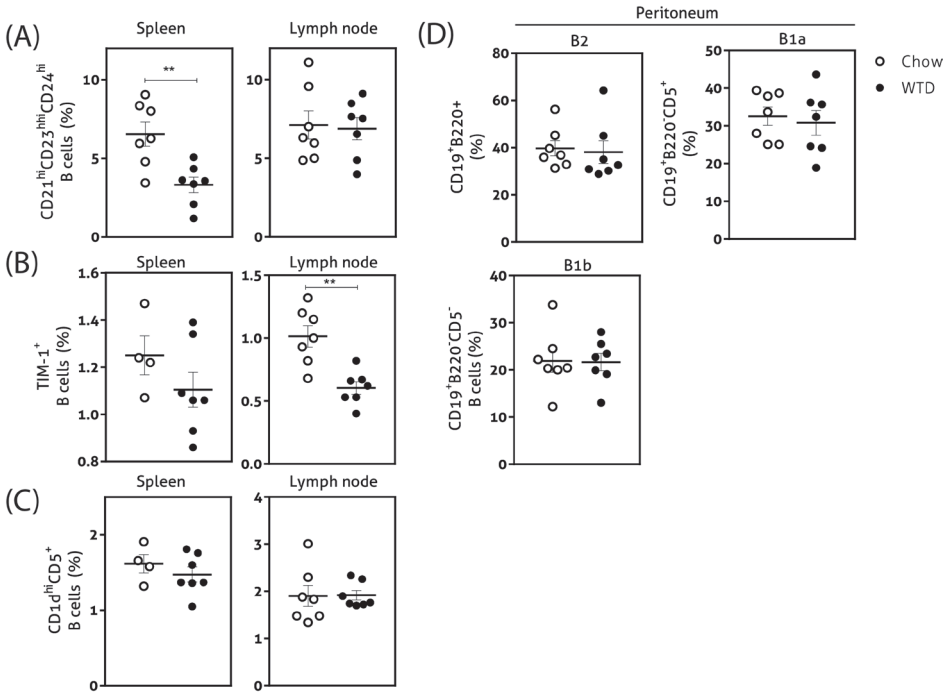


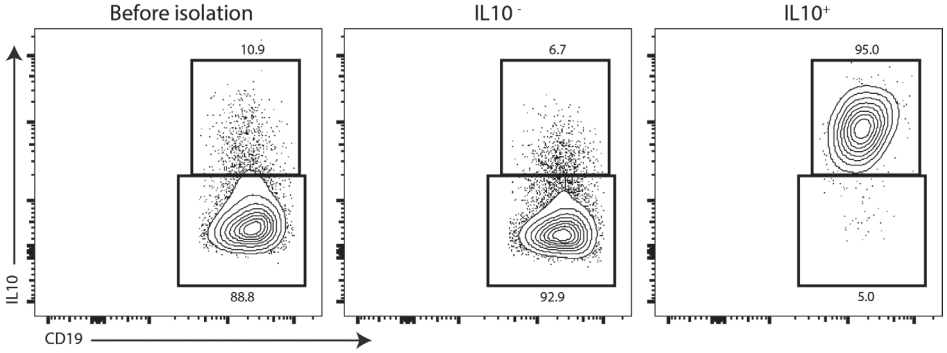
Supplementary Figure S1. Representative gating of IL-10^{+} B cells. Splenocytes were isolated and stimulated with a combination of LPS (50 µg/ml), PMA (50 ng/ml), ionomycin (500 ng/ml) and monensin (2 µM) for 5 hours. Fc receptors were blocked and samples were stained with anti-mouse fluorochrome conjugated antibodies against B220, CD19 and IL-10. After dead cells were excluded, B220^{+} cells were gated followed by gates for IL-10 and CD19 which were set according to unstimulated controls or to isotype controls.



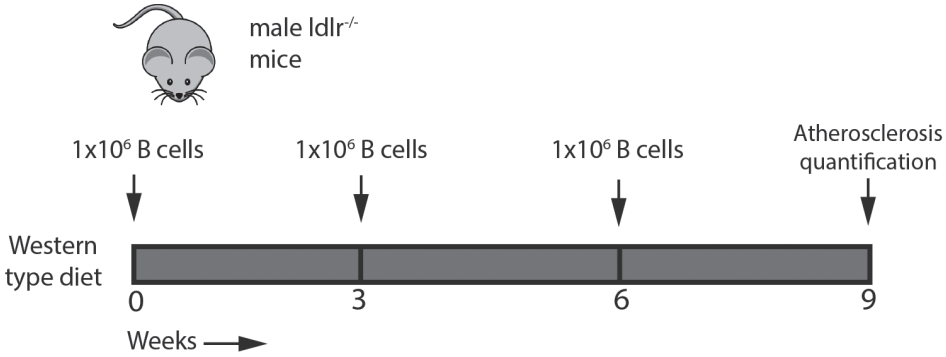
Supplementary Figure S2. IL-10^{+} B cells in the blood and non-draining lymph nodes are not affected by Western Type Diet (WTD). Mice were either put on WTD or normal chow diet for 9 weeks after which single cell suspensions of blood, mesenteric lymph nodes and Peyer's patches were stimulated with LPS+PMA+Ionomycin and Monensin for 5 hours. Fc receptors were blocked and samples were stained with anti-mouse fluorochrome conjugated antibodies against B220, CD19 and IL-10. Gates were set according to unstimulated controls or to isotype controls.



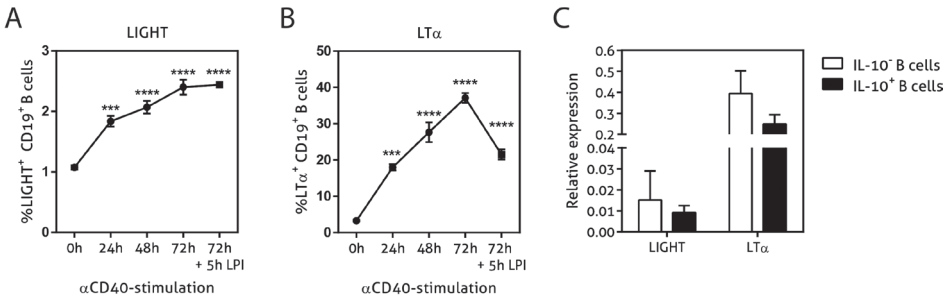
Supplementary Figure S3. Regulatory B cell subsets. *Ldlr*$^{-/-}$ mice were fed a WTD or normal chow diet for 9 weeks after which spleens and lymph nodes were analyzed with flow cytometry to determine **(A)** CD21^{hi}CD23^{hi}CD24^{hi} B cells, **(B)** TIM-1^{+} B cells and **(C)** CD1d^{hi}CD5^{+} B cells. **(D)** Single cell suspensions from the peritoneum were analyzed for B2 (CD19^{+}B220^{+}), B1a (CD19^{+}B220^{-}CD5^{+}) and B1b (CD19^{+}B220^{-}CD5^{-}) cells.



Supplementary Figure S4. Isolation of pure and viable IL-10^{+} B cells. B cells were cultured with an agonistic CD40 antibody (5 µg/ml) for 72 hours after which they were stimulated with LPS (50 µg/ml), PMA (50 ng/ml) and ionomycin (500 ng/ml) to secrete IL-10. Using a specialized IL-10-catching antibody, IL-10-secreting B cells were separated from non IL-10-secreting B cells using magnetic beads. Flow cytometry analysis of the isolated cells showed that this consistently resulted in a ≥95% purity of viable IL-10^{+} B cells.



Supplementary Figure S5. Experimental setup of adoptive transfer experiment. *Ldlr*$^{-/-}$ mice were put on a Western type diet for 9 weeks and received either three i.v. injections of PBS, IL-10$^+$ or IL-10$^-$ B cells (1x10^6 cells/injection) after which the mice were sacrificed and atherosclerosis was quantified.



Supplementary Figure S6. CD40-stimulation of B cells leads to increased lymphotoxin-alpha and LIGHT expression. Isolated CD19$^+$ B cells were stimulated for indicated periods of time with an agonistic CD40 antibody (5 µg/ml) with additional LPS, PMA and ionomycin stimulation where indicated. Subsequently, expression of (A) LIGHT and (B) lymphotoxin-alpha (LTα) were measured with flow cytometry. (C) IL-10$^-$ and IL-10$^+$ CD19$^+$ B cells were isolated after stimulation for 72 hours with an agonistic CD40 antibody followed by LPS, PMA and ionomycin stimulation for 5 hours. Subsequently, the expression of LIGHT and lymphotoxin-alpha (LTα) were measured with qPCR. Data are shown as mean ± SEM (***p<0.001, ****p<.00001).